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ANTAGONISTIC POTENTIAL OF FLUORESCENT *Pseudomonas* AND ITS IMPACT ON GROWTH OF TOMATO CHALLENGED WITH PHTOPATHOGENS

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ABSTRACT

This study focused on the antagonistic potential of fluorescent *Pseudomonas in vitro*, and its inoculation effect on growth performance of *Lycopersicon esculentum* in *Fusarium oxysporum* and *Rhizoctonia solani* infested soil. Biochemical characteristics of fluorescent *Pseudomonas* showed that all ten isolates were positive to catalase, amylase, gelatinase and siderophore production. While three isolates (Pf5, Pf6 and Pf9) were oxidase positive, nine isolates (Pf1, Pf2, Pf3, Pf4, Pf6, Pf7, Pf8, Pf9, and Pf10) were tolerant to 6.5% NaCl. Isolates Pf5 and Pf6 were resistant to all the test antibiotics; in contrast, the remaining eight isolates responded differently to different antibiotics. Isolates Pf5 and Pf6 were antagonistic against 14 bacterial species, and two pathogenic fungi (*F. oxysporum* and *R. solani*). Inoculation with fulorescent *Pseudomonas* Pf5 induced a significant increase in root and shoot length, and dry weight. Treatment of plants with either *F. oxysporum* or *R. solani* drastically reduced the root and shoot length and dry weight of the plant. However, in the presence of fluorescent *Pseudomonas* the adverse effect of the pathogens on growth of *L. esculentum* was alleviated.

Key Words: Amylase, gelatinase, *Lycopersicon esculentum*, phytopathogens

RÉSUMÉ

Cette étude a porté sur le potentiel antagonistique du *Pseudomonas* fluorescent, *in vitro* et les effets de son inoculation sur la performance en croissance du *Lycopersicon esculentum* dans le sol infesté par le *Fusarium oxysporum* et le *Rhizoctonia solani*. Les caractéristiques biochemiques du *Pseudomonas* fluorescent ont montré que tous les dix isolats étaient positives eu égard à la production de catalase, amylase, gélatinase et sidérophore. Alors que trois isolats (Pf5, Pf6 and Pf9) étaient oxidase positifs, neuf isolats (Pf1, Pf2, Pf3, Pf4, Pf6, Pf7, Pf8, Pf9, et Pf10) étaient tolerant au 6.5% NaCl. Les isolats Pf5 et Pf6 étaient résistants à tous les test antibiotiques; au contraire, les huit isolats restants ont répondu différemment aux différents antibiotiques. Les isolats Pf5 et Pf6 étaient antagonistiques contre 14 espèces de bactéries, et deux champignons pathogeniques (*F. oxysporum* et *R. solani*). L'inoculation avec *Pseudomonas* fulorescent Pf5 a induit une augmentation significative des raciness et de la longueur des tiges, ainsi que du poids sec. Le traitement de plants avec du *F. oxysporum ou du R. solani* ont radicalement réduit la longueur des raciness et tiges ainsi que le poids sec du plant. Cependant, en présence du *Pseudomonas* fluorescent, l'effet adverse du pathogène sur la croissance du *L. esculentum* était allevié.

Mots Clés: Amylase, gélatinase, *Lycopersicon esculentum*, phytopathogènes

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that actively colonise roots and stimulate plant growth either directly or indirectly. Direct stimulation of plant growth takes place by providing phytohormones (Mordukhova *et al*., 1991) or by solubilisation of mineral phosphate and other nutrients (Glick, 1995), while indirect stimulation takes place through suppression of phytopathogens by the production of siderophores (Scher and Baker, 1982) or by producing antibiotics (Thomashow and Weller, 1996). The rhizobacteria that control soil-borne pathogens are called biocontrol rhizobacteria.

Fusarium oxysporum causes foot and root rot in tomato plants and is a serious problem for both field and greenhouse crops (Jarvis, 1988). Chemical pesticides do not effectively suppress the rot (Benhamou *et al*., 1994). Although the use of *Fusarium*-resistant tomato cultivars can control the disease to some extent, the development of new pathogenic races is a challenge. Biological control is an alternate and effective strategy to manage the disease. Among the biocontrol rhizobacteria, *Psudomonas*, *Burkholderia* and *Bacillus* spp. are the important ones (Weller, 1988; Weller and Cook, 1983) including *Fusarium* spp. (Van Peer *et al*., 1991; Liu *et al*., 1995).

It has been suggested that microorganisms isolated from the root or rhizosphere of a particular crop may provide better control of diseases than organisms originally isolated from other plant species (Cook, 1993). Therefore, screening of locally adapted biocontrol strains is essential to get desired results.

The objective of this study was to isolate fluorescent *Pseudomonas* from tomato rhizosphere soils in South Tamil Nadu and assess their antagonistic potential against target bacteria and pathogenic fungi *in vitro*, as well as to know the impact of inoculation with fluorescent *Pseudomonas* on growth performance of tomato challenged with *F. oxysporum* and *R. solani*.

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MATERIALS AND METHODS

Fluorescent *Pseudomonas* was isolated from tomato rhizosphere soil collected from Madurai, Dindigul and Virudhunagar districts in India by dilution plate technique (Wollum, 1982) using King's B medium (King *et al*., 1954). The isolates were named as follows:

Isolate of fluorescent *Pseudomonas* from Peraiyur, Madurai district (Pf1);
Isolate of fluorescent *Pseudomonas* from Nilakottai, Dindigul district (Pf2);
Isolate of fluorescent *Pseudomonas* from Silukuwarpatti, Dindigul district (Pf3);
Isolate of fluorescent *Pseudomonas* from Vadipatti, Madurai district (Pf4);
Isolate of fluorescent *Pseudomonas* from Mallapuram, Madurai district (Pf5);
Isolate of fluorescent *Pseudomonas* from Alagapuri, Virudhunagar district (Pf6);
Isolate of fluorescent *Pseudomonas* from Uthapuram, Madurai district (Pf7);
Isolate of fluorescent *Pseudomonas* from Kallupatti, Madurai district (Pf8);
Isolate of fluorescent *Pseudomonas* from Kottaipatti, Madurai district (Pf9); and
Isolate of fluorescent *Pseudomonas* from Elumalai, Madurai district (Pf10).

The isolates were tested for Gram reaction, catalase reaction (Graham and Parker, 1964) by the formation of effervescence from 5 days old slant culture due to the addition of few drops of 3% H_2O_2, and gelatin liquefaction (Hirsch *et al*., 1980) by stabbing the gelatin slants with the cultures and incubation at 30° C for 15 days. Oxidase reaction was tested by placing High Media oxidase discs (Code No. DD 018) on individual colonies and observing blue colour formation in the disc within 5 to 10 seconds. Starch hydrolysis was determined by flooding the 5 days old culture in starch agar with Lugol's iodine solution. Salt tolerance was assessed by observing the growth of the culture in King's B medium supplemented with 6.5% NaCl.

Siderophores were assayed by $FeCl_3$ reaction (Snow, 1954) for general types (appearance of orange or reddish brown colour upon mixing 0.5 ml of culture filtrate and 0.5 ml of 2% $FeCl_3$ solution), Arnows reaction (Arnow, 1937) for catechol type (appearance of red colour upon mixing 1 ml of culture filtrate, 1 ml of nitrite molybdate reagent and 1 ml of 1 N NaOH), and tetrazolium reaction (Snow, 1954) for hydroxamate type (appearance of deep red colour upon adding a pinch of tetrazolium salt to a mixture containing 2 drops 2N NaOH and 0.1 ml of culture filtrate) . Quantification of hydroxamate type of siderophores was done by Csaky test (Csaky, 1948). A mixture containing 2 ml of culture filtrate and 2 ml of 3M sulphuric acid was autoclaved for 4 hr. To the hydrolysed solution, 7 ml of 2M sodium acetate, 2 ml of sulphanilamide (1% in 30% acetic acid) and 2 ml of iodine solution (0.65% iodine in 1% KI) were added. The mixture was swirled for 5 min and the excess iodine removed by addition of 2 ml of 1.5% sodium arsenite and 2 ml of 0.05% naphthyl ethylene diamine. The resultant solution was left at 30° C for 30 min, made to 50 ml with distilled water and read at 543 nm. Hydroxylamine hydrochloride was used as standard.

Antibiotic sensitivity test was carried out by standardised filter paper disc agar diffusion method of Bauer *et al*. (1966). King's B agar medium was seeded with fluorescent *Pseudomonas*. Then, antibiotic discs with known antibiotic concentration, *viz*. Penicillin (PEN), Streptomycin (STR), Gentamicin (GEN), Kanamycin (KAN), Rifampin (RIF), Tetracycline (TET), Ampicillin (AMP) and Chloramphenicol (CHL) obtained from HiMedia, Mumbai were placed aseptically on the surface of the agar plates. The plates were incubated at 30° C for 2 days before the inhibition zone diameter was measured.

Antagonistic potential of fluorescent *Pseudomonas* against 15 bacterial species, *viz. Klebsiella* sp., *Zymomonas mobilis, Xygomonas* sp., *Streptococcus* sp., *Staphylococcus aureus, Micrococcus luteus, Shigella* sp., *Escherichia coli, Bacillus subtilis, Proteus vulgaris, Azotobacter* sp., *Lactobacillus* sp., *Salmonella typhi, Cellulomonas* sp., and *Vibrio cholera* was determined by agar bioassay. The target culture (0.1 ml of 24 hr old culture) was mixed with 20 ml of sterile molten nutrient agar cooled to 45° C. The inoculated agar medium was poured into Petri-plates and wells were punched with a 5 mm diameter gel puncher. A 25 µl volume of antagonistic cultures (Pf5 or Pf6) was poured into each well. A well with sterile nutrient broth alone served as control. Plates were incubated for 1 day at 5° C, followed by 2 days at 30° C. Inhibition of target organisms was measured as inhibition zone diameter in cm around the well.

In vitro antibiosis against phytopathogenic fungi by fluorescent *Pseudomonas* was tested by dual culture technique. A loopful of fluorescent *Pseudomonas* culture (24 hr old) was streaked on one side of the potato dextrose agar (PDA) plates. Mycelial discs (6 mm in diameter) of *Fusarium oxysporum* and *Rhizoctonia solani* cut from an actively growing culture were placed on the opposite side to the bacteria. The plates were incubated at 30° C for 7 days. The zone of inhibition of radial growth of the pathogen (a clear zone between the edges of fungal mycelia and the bacterial streak) was measured using a pair of calipers. Control plates contained only fungal culture.

Seeds of *L. esculentum* were surface sterilised with 0.1% $HgCl_2$ for 2 min and bacterised with fluorescent *Pseudomonas* Pf5. *Fusarium oxysporum* and *R. solani* were cultivated in natural medium (sorghum seeds were soaked in 1% sucrose solution for 16 hr and autoclaved for 45 min) and the fungus cultures were incorporated into the sterile soil-sand mixture (2:1 ratio) in earthen pots. The following treatment schedule was followed.

Uninoculated control (T0);
Fluorescent *Pseudomonas* inoculation (T1);
F. oxyporum inoculation (T2);
R. solani inoculation (T3);
fluorescent *Pseudomonas* + *F. oxysporum* inoculation (T4); and
Fluorescent *Pseudomonas* + *R. solani* inoculation T5

Plants were grown in a greenhouse under conditions of broad day light and watered with sterile tap water. Eighteen day-old plants were harvested and root and shoot lengths recorded.

Plant materials were cut into bits and dried in an oven at 90° C for 3 days and dry weight was determined. The data were subjected to statistical analysis by using the COSTAT package for one – way ANOVA and Newman Keuls test.

RESULTS AND DISCUSSION

All the isolates of fluorescent *Pseudomonas* were positive to catalase, amylase, gelatinase and siderophore production (Table 1). The siderophores were of hydroxamate type. Among the ten isolates, Pf5 produced maximum quantity of siderophores (866 µg mg^{-1} protein). Oxidase test showed a positive reaction only to three isolates, *viz* pf5, pf6 and Pf9. With the exception of Pf5, all the other 9 isolates were tolerant to 6.5% NaCl.

Innate antibiotic resistance test indicated that Pf5 and Pf6 were resistant to the eight antibiotics *viz* Penicillin (PEN), Streptomycin (STR), Gentamicin (GEN), Kanamycin (KAN), Rifampin (RIF), Tetracycline (TET), Ampicillin (AMP) and Chloramphenicol (CHL) (Table 2). However, for the remaining 8 isolates, the extent of antibiotic resistance differed with different antibiotics. On the basis of innate antibiotic resistance, 2 isolates of fluorescent *Pseudomonas* Pf5 and Pf6 were selected for their antagonistic potential against 15 bacterial species and 2 pathogenic fungal species.

Among the 15 bacterial species, 14 species *viz Klebsiella* sp., *Xygomonas* sp., *Streptococcus* sp., *Staphylococcus aureus*, *Micrococcus luteus*, *Shigella* sp., *Escheritia coli*, *Bacillus subtilis*, *Proteus vulgaris*, *Agrobacter* sp., *Lactobacillus* sp., *Salmonella typhi*, *Cellulomonas* sp., and *Vibrio cholera* were sensitive to Pf5 and pf6. *Zymomonas mobilis* was the only species that could resist the antagonistic action of Pf5 and Pf6 (Table 3). Further, based on inhibition zone formation, *Bacillus subtilis* was the most sensitive organism, while *Xygomonas* sp., was the least sensitive organism. *Pseudomonas* spp., isolated from spoiled and fresh fish have been shown to possess a broad antibacterial potential which is siderophore-mediated (Gram, 1993). The two isolates, Pf5 and Pf6, exhibited antagonistic activity against pathogenic fungi *F. oxysporum* and *R. solani in vitro*. The isolates induced

TABLE 1. Biochemical characteristics of fluorescent *Pseudomonas*

Characteristics	Isolates									
	Pf 1	Pf 2	Pf 3	Pf 4	Pf 5	Pf 6	Pf 7	Pf 8	Pf 9	Pf 10
Gram staining	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+
Amylase	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	+	+	-	-	+	-
Salt tolerance(6.5% NaCl)	+	+	+	+	-	+	+	+	+	+
Gelatinase	+	+	+	+	+	+	+	+	+	+
Siderophores* (hydroxamate type (µg mg^{-1} protein)	740	800	630	695	867	825	720	771	785	643

+ sign indicates positive response; – sign indicates negative response; * Siderophore values represent average of three replications

TABLE 2. Innate antibiotic resistance of fluorescent *Pseudomonas*

Isolates	Antibiotics inhibition zone diameter (cm)							
	PEN 10 units disc^{-1}	STR 10 mcg disc^{-1}	GEN 10 mcg disc^{-1}	KAN 30 mcg disc^{-1}	RIF 5 mcg disc^{-1}	TET 30 mcg disc^{-1}	AMP 10 mcg disc^{-1}	CHL 50 mcg disc^{-1}
Pf 1	-	+(1.5)	+(1.6)	+(1.5)	+(0.8)	+(1.4)	+(1.8)	-
Pf 2	-	+(1.6)	+(1.7)	+(1.7)	+(0.8)	+(0.8)	+(1.5)	-
Pf 3	-	+(1.8)	+(1.6)	+(1.8)	+(0.8)	+(0.7)	+(1.4)	-
Pf 4	-	+(1.1)	+(1.2)	+(1)	+(0.8)	+(1.6)	+(1.5)	-
Pf 5	-	-	-	-	-	-	-	-
Pf 6	-	-	-	-	-	-	-	-
Pf 7	-	+(1.0)	+(0.9)	+(0.9)	+(0.8)	+(1)	+(0.7)	-
Pf 8	-	+(0.7)	+(0.7)	+(1.3)	-	-	+(0.7)	-
Pf 9	-	+(0.9)	+(1)	+(1.7)	+(0.1)	+(0.8)	+(0.9)	-
Pf 10	-	+(0.9)	+(1.1)	+(0.9)	+(0.9)	+(0.7)	+(1.5)	-

+ sign indicates antibiotic sensitivity; – sign indicates antibiotic resistance; Values represent average of three replications

TABLE 3. Antagonistic potential of fluorescent *Pseudomonas* against some bacterial and fungal species

Target organism	Inhibition zone diameter (cm) Pf 5	Inhibition zone diameter (cm) Pf 6
Klebsiella sp.	1.6	2.1
Zymomonas mobilis	-	-
Xygomonas sp.	0.9	0.9
Streptococcus sp.	1.0	1.0
Staphylococcus aureus	1.5	1.3
Micrococcus luteus	1.3	1.2
Shigella sp.	1.6	2.1
Escherichia coli	1.2	0.8
Bacillus subtilis	2.2	2.3
Proteus vulgaris	2.2	1.7
Azotobacter sp.	1.1	1.3
Lactobacillus sp.	1.6	1.5
Salmonella typhi	1.8	1.3
Cellulomonas sp.	1.3	2.0
Vibrio cholera	1.7	1.6
Fusarium oxysporum	1.2 to 3.8 cm*	1.1 to 3.3 cm*
Rhizoctonia solani	0.3 to 0.9 cm*	0.3 to 0.9 cm*

Values represent average of three replications. *Distance between the edges of fungal mycelia and the bacterial streak

inhibition zones ranging from 0.3 to 3.8 cm towards the phytopathogenic fungi. However, maximum inhibition was shown by Pf5. Similar antifungal activity exhibited by fluorescent pseudomonads was reported by Cook *et al*. (1995) and Chythanya *et al.* (2002). The isolate Pf5 was tested on *L. esculentum* challenged with phytopathogens to assess its biocontrol potential *in vivo*.

Inoculation with fluorescent *Pseudomonas* induced a significant increase in root and shoot length (123 and 96%, respectively) over the uninoculated control (Table 4). In contrast, pathogen treatment caused a reduction in root

TABLE 4. Impact of fluorescent *Pseudomonas* Pf5 inoculation on root and shoot length and dry matter accumulation in 18 day-old *L. esculentum* grown in pathogen infested soil

Treatment	Root length (cm plant^{-1})	Shoot length (cm plant^{-1})	Root dry weight (g plant^{-1})	Shoot dry weight (g plant^{-1})
Uninoculated control	4.16 b ± 0.76	12.83 b ± 0.28	0.016 bc ± 0.005	0.057 ab ± 0.005
Fluorescent *Pseudomonas*	9.30 a ± 1.58	25.16 a ± 4.25	0.027 a ± 0.004	0.103 a ± 0.030
F. oxysporum	3.16 b ± 0.28	9.83 b ± 0.76	0.010 c ± 0.002	0.020 b ± 0.003
R. solani	4.00 b ± 0.86	11.16 b ± 0.86	0.010 c ± 0.002	0.022 b ± 0.004
Fluorescent *Pseudomonas* + *F. oxysporum*	8.36 a ± 0.76	22.33 a ± 1.15	0.024 ab ± 0.004	0.075 a ± 0.020
Fluorescent *Pseudomonas* + *R. solani*	8.5 a ± 1.32	24.50 a ± 4.44	0.022 ab ± 0.004	0.090 a ± 0.030
L.S.D. (P<0.05)	1.810***	4.621***	0.0065***	0.0347***

± Standard deviation. Values suffixed with different letters on the same column indicate significant differences. *, **, *** = Extent of significance; NS = Not significant

and shoot length (4 to 24% and 13 to 23%, respectively). Pathogen-induced reduction in root and shoot length was nullified by fluorescent *Pseudomonas* inoculation. That is, the levels of root and shoot length in fluorescent *Pseudomonas* + pathogen infested plants reached next only to that of fluorescent *Pseudomonas* alone inoculated plants (100 to 104% increase in root length and 74 to 91% increase in shoot length over the control). *Pseudomonas fluorescens* has been shown to increase seed germination, root and shoot length, and seedling vigour in several instances (Ramamoorthy *et al*., 2001; Khalid *et al*., 2004; Egamberdieva, 2008).

It is clear in Table 4 that fluorescent *Pseudomonas* inoculation enhances root and shoot biomass (69 and 81% increase, respectively), compared to the control. On the contrary, pathogen treatment resulted in 38% reduction in root biomass and 61 to 65% reduction in shoot biomass. However, in the presence of fluorescent *Pseudomonas*, the pathogen-induced effect on root and shoot biomass was alleviated (38 to 50% higher root biomass and 32 to 58% higher shoot biomass over the control).

Mahnaz and Lazarovits (2006) reported an increase in root and shoot weight of corn plants upon inoculation with a strain of *Pseudomonas putida* that also possess antagonistic activity against *Fusarium*. Production of siderophores is an important trait of PGPR that may indirectly influence the plant growth. Siderophore producing biocontrol agents have competitive advantage over the pathogens for the absorption of available iron. *Pseudomonas fluorescens* and *P. putida* have been shown to be successful seed inoculants for siderophore-mediated biocontrol agents for several plant pathogens (Dileep Kumar and Dube, 1972; Van Peer *et al*., 1990). Thus, Pf5 the powerful siderophore producer (866 µg mg^{-1} protein) has the potential to suppress the activity of pathogens and enhance the plant growth.

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